Localization of the neuropeptide NGIWYamide in the holothurian nervous system and its effects on muscular contraction

Masahide Inoue[1]**, Rüdiger Birenheide**[1]**, Osamu Koizumi**[2]**, Yoshitaka Kobayakawa**[3]**, Yojiro Muneoka**[4] **and Tatsuo Motokawa**[1]*

[1]*Basic Biology, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Meguro-ku, Tokyo 152-8551, Japan*
[2]*Neuroscience Laboratory, Faculty of Human Environmental Science, Fukuoka Women's University, Fukuoka 813-8529, Japan*
[3]*Biological Laboratory, Faculty of Science, Kyushu University, Fukuoka 810-8560, Japan*
[4]*Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8526, Japan*

NGIWYamide is a peptide recently isolated from the sea cucumber *Apostichopus japonicus*. It stiffens the connective tissue of the holothurian body wall. Localization of NGIWYamide was investigated by immunohistochemical staining with antiserum raised against NGIWYamide. In holothurian nervous systems NGIWYamide-like immunoreactivity (NGIWYa-LI) was observed in the hyponeural and ectoneural regions of the radial nerve cord, as well as in the circumoral nerve ring, podial nerves, tentacular nerves, the basiepithelial nerve plexus of the intestine and in cellular processes running through the body wall dermis. Labelled nerve fibres from the hyponeural part of the radial nerve running towards the circular muscle and from the podial nerve into the body wall dermis suggest that NGIWYamide controls both muscle and connective tissue. We examined the effect on muscle activity of the sea cucumber. NGIWYamide (10^{-7} to 10^{-4} M) caused contraction of the longitudinal body wall muscle. Tentacles showed contraction only at a higher dose (10^{-4} M). NGIWYamide (10^{-4} M) inhibited spontaneous contraction of the intestine.

Keywords: neuropeptide; immunohistochemistry; echinoderm nervous system; sea cucumber

1. INTRODUCTION

The nervous systems of echinoderms, in particular holothurians, have been poorly investigated. This seems due to the difficulty in nerve cell identification and problems with access to nerve cells for electrophysiology due to their small size, because they are often embedded in calcareous ossicles or dense connective tissue. In holothurians, signal transmission remains a mystery because synapses have never been found or described. So far, acetylcholine (ACh) is the only classical neurotransmitter shown to have an effect; its site of action, however, is unknown (Cobb 1987). Besides classical transmitters, neuropeptides seem to play some role in signal transmission and modulation. GFSKLYFamide was isolated from the digestive tract of the sea cucumber *Holothuria glaberrima* (Diaz-Miranda *et al.* 1992) and its presence in the nervous system and other organs was shown by immunohistochemistry (Diaz-Miranda *et al.* 1995). It induces relaxation of the intestine and the longitudinal muscle in the body wall of sea cucumbers (Diaz-Miranda & Garcia-Arraras 1995). This is the only neuropeptide with a known effect and localization so far described for holothurians.

Recently, we isolated four new peptides from the body wall of the sea cucumber *Apostichopus japonicus*. They are NGIWYamide, holokinin 1, holokinin 2 and stichopin, which all affect the mechanical properties of the dermis of the sea cucumber body wall (Iwakoshi *et al.* 1995; Birenheide *et al.* 1998). The dermis is a catch connective tissue that can change its mechanical properties dramatically in response to various stimuli under nervous control (Motokawa 1981, 1984; see also Ruppert & Barnes 1994; Schmidt-Nielsen 1997). NGIWYamide stiffens the dermis, whereas holokinins soften it and stichopin blocks the effect of ACh (Birenheide *et al.* 1998). In the present study, we have generated antisera against NGIWYamide to examine the distribution and localization of this peptide by indirect immunohistochemistry. We also showed that NGIWYamide affects muscle activity in the sea cucumber.

2. MATERIAL AND METHODS

Specimens of the sea cucumber *A. japonicus* were collected near Noto Marine Laboratory of the University of Kanazawa. The animals were kept in circulating seawater aquaria at 18 °C.

(a) *Production of NGIWYamide antiserum*
(i) *Antibody production*

Polyclonal antibodies recognizing NGIWYamide were produced by conjugating a synthetic sequence, CNGIWYamide, via succinimidyl *m*-maleimidebenzoate (MBS; Peerters *et al.*

1989), to keyhole limpet haemocyanin (KLH). The conjugate (100 μg peptide per animal) containing complete Freund's adjuvant (Difco) was initially injected into two New Zealand white rabbits and, thereafter, the conjugate (100 μg peptide per animal), together with incomplete Freund's adjuvant (Difco), was injected five times. During immunization, the antibody titre was monitored by an enzyme-linked immunosorbent assay (ELISA). After the final boost, blood was collected from the ear vein for serum preparation. The serum was absorbed with 1 mg ml^{-1} of KLH overnight at 4 °C and then centrifuged. The supernatant was passed through a membrane filter (Millipore, sterile, low protein-binding type, 0.45 mm filter unit) and 0.1% sodium azide was added. Aliquots of the filtrate were stored at −80 °C.

(ii) *ELISA of NGIWYamide*

The CNGIWYamide peptide (2.5 μg ml^{-1} and 50 ml per well) was bound to the well of a high-binding type ELISA plate (Nunc-Immuno Plate, Maxi Sorp F96 Nunc, Denmark). A kit for ELISA staining (Microwell ELISAmate for Peroxidase Conjugate, Kirkegaard & Perry Laboratories, USA) was used. Plates were incubated overnight at 4 °C with anti-NGIWYamide antiserum (various dilutions and 100 ml per well). As a second antibody, peroxidase anti-rabbit IgG(H + L) (Vector Laboratories, USA, 1:500 dilution and 50 ml per well) was used. Plates were read at OD405 with a microplate reader (Immuno mini NJ-2300, Intermed, Japan). After the fifth immunization the titre of CNGIWYamide immunoreactivity was sufficiently high (1:6250 dilution was positive).

(b) *Immunohistochemistry*

Animals were anaesthetized in 1% menthol in artificial seawater (ASW, Jamarin Laboratory, Japan). Dermis tissues, including the radial nerve cord, the circular muscle and the longitudinal muscle of the body wall and samples of the anterior region, which included the circumoral nerve ring and tentacles, as well as samples of the intestine, were dissected and immediately fixed with Bouin's fluid for 2–4 h at room temperature. Specimens were dehydrated in an ethanol series and embedded in Paraplast Plus (Sigma Aldrich, Japan). Sections of 7–10 μm thickness were cut and mounted on poly-L-lysine-coated slides. After dewaxing and rehydrating with phosphate-buffered saline (PBS, Nissui Pharmaceutical, Japan) sections were immersed in blocking buffer (1% bovine albumin in PBS) for 1 h at room temperature before incubation overnight at 4 °C with primary antisera against NGIWYamide diluted in PBS (1:1000–1:2000). After three washes in PBS, the sections were incubated for 1 h in peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, USA) diluted in PBS (1:1000) and then in peroxidase anti-peroxidase (Sigma Aldrich, Japan) diluted in PBS (1:400) at room temperature. The immunostaining product was visualized with 0.03% 3′, 3-diaminobenzidine (Nakalai Tesque, Japan) and 0.01% hydrogen peroxide in PBS. Developing time was 3–5 min.

Preabsorption controls were carried out to determine the specificity of the primary antiserum. NGIWYamide antisera diluted 1:1000 were incubated with 10^{-3} M and 10^{-6} M synthetic NGIWYamide overnight at 4 °C.

To investigate the organization of the nervous system, samples were dissected, fixed and cut in the same way as described above. Deparaffinized and rehydrated sections were then stained with Milligan trichrome (Humason 1979).

(c) *Pharmacological tests*

The longitudinal muscle of the body wall was dissected out from *A. japonicus* and cut into strips of 15 mm × 3 mm × 3 mm. Tentacles were isolated from the buccal part and used whole. The intestine was dissected and cut into sections of *ca*. 15 mm length.

The contraction forces were measured. The sample was fixed to a holder in an experimental trough filled with 1 ml ASW. The other side of the sample was connected to an isometric force transducer (LVS-20GA, KYOWA, Japan) via a silver chain. Temperature was controlled via a water bath at *ca*. 18 °C. Synthetic NGIWYamide dissolved in ASW was added to the experimental trough filled with ASW to a final concentration of 10^{-8} to 10^{-4} M. After the reaction, NGIWYamide was washed out with a constant flow of ASW.

3. RESULTS

(a) *Gross morphology*

The outer surface of the body wall of *A. japonicus* is covered with a thin layer of cuticle. The epidermis overlies a thick dermis, which occupies most of the thickness of the body wall. Towards the body cavity the dermis is lined with a thin layer of circular muscle. The layer does not form a complete circle: longitudinal muscles and radial nerve cords interrupt the circle at each ambulacrum.

The nervous system follows the usual echinoderm pattern with a circumoral nerve ring and five radial nerve cords. The circumoral nerve ring encircles the mouth close to the base of the tentacles. Five radial nerve cords extend from the ring towards the posterior of the animal. The tentacular nerves arise from the anterior edge of the nerve ring (see figure 1a).

Radial nerves are composed of a thicker outer ectoneural and a thinner inner hyponeural part. These are morphologically separated by a thin partition of connective tissue (see figure 2a). The podial nerves with the epineural sinus emerge from the ectoneural part of the radial nerve and run along each tube foot (see figure 1b).

Unlike the radial nerve, the circumoral nerve ring has only one neural part (see figure 2b). This is ectoneural because there is no apparent partitioning between the neural part of the circumoral nerve ring and the ectoneural part of the radial nerve (see figure 2c). The connective tissue that divides the ectoneural from the hyponeural part in the radial nerve is continuous with the connective tissue that covers the circumoral nerve ring. Most of the cell bodies in the circumoral nerve ring are found in the periphery of the nerve (see figure 2b).

(b) *Immunohistochemistry*

(i) *Radial nerve and circumoral nerve ring*

NGIWYamide-like immunoreactivity (NGIWYa-LI) was abundant in the radial nerve cord and circumoral nerve ring. Immunohistochemical staining of cross-sections revealed the presence of NGIWYa-LI in both the ectoneural and hyponeural parts of the radial nerve cord (see figure 3a). In the ectoneural part, immunoreactive cell bodies are located in the periphery of the nerve (see figure 3c) and adjacent to the connective tissue layer separating the hyponeural and ectoneural parts (see figure 3d). Dense varicosities and nerve fibres expressing NGIWYa-LI could be seen in the ectoneural part and were also found in the hyponeural part (see figure 3d,e).
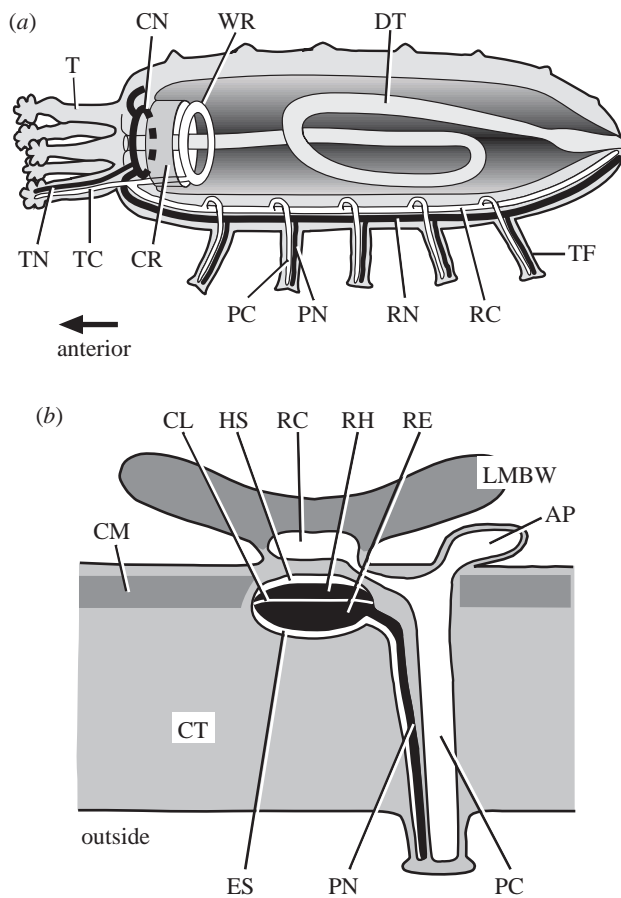
Figure 1. The holothurian nervous system. (*a*) Diagram showing the nervous system, water vascular system and digestive tract in lateral view. (*b*) Cross-section of the radial nerve cord. The podial nerve extends from the ectoneural part of the radial nerve cord. AP, ampulla; CL, connective tissue layer; CM, circular muscle; CN, circumoral nerve ring; CR, calcareous ring; CT, connective tissue of the body wall; DT, digestive tract; RE, ectoneural part of the radial nerve; ES, epineural sinus; RH, hyponeural part of the radial nerve; HS, hyponeural sinus; LMBW, longitudinal muscle of the body wall; PC, podial water canal; PN, podial nerve; RC, radial water canal; RN, radial nerve; T, tentacle; TC, tentacular water canal; TF, tube foot; TN, tentacular nerve; WR, water ring canal.

Figure 2. Cross-section of the radial nerve cord and circumoral nerve ring stained by the Milligan trichrome method. (*a*) Radial nerve cord consisting of the hyponeural and ectoneural nerves separated by a connective tissue layer; scale bar, 50 μm. (*b*) Circumoral nerve ring consisting only of the ectoneural nerve; scale bar, 100 μm. (*c*) Connection of the radial nerve and circumoral nerve ring. The radial nerve (to the left) consists of two neural parts, the hyponeural and ectoneural parts; the latter is continuous with the circumoral nerve ring (to the right); scale bar 200 μm. Abbreviations as in figure 1.

However, immunoreactive cell bodies were not seen in the hyponeural part.

NGIWYa-LI was detected in the circumoral nerve ring (see figure 3*b*). Immunoreactive cell bodies were located in the mid portion (see figure 3*f*) and the periphery of the nerve. Dense varicosities and nerve fibres expressing NGIWYa-LI could be also observed.

Branches containing NGIWYa-LI labelled varicose nerve fibres emerged from the hyponeural part of the radial nerve and extended towards the circular body wall muscles (see figure 3*g*). Serial sections revealed that branches emerged at certain intervals along the length of the radial nerve. Labelled cell bodies in the coelomic epithelium were observed at the edge of the hyponeural part of the radial nerve cord just where the branch emerged. These cells had axon-like processes extending towards the hyponeural part or towards the circular muscles (see figure 3*h*). Labelled fibres ran in close apposition to and in parallel with the length of the circular muscles (see figure 3*i*). The fibres were probably a part of
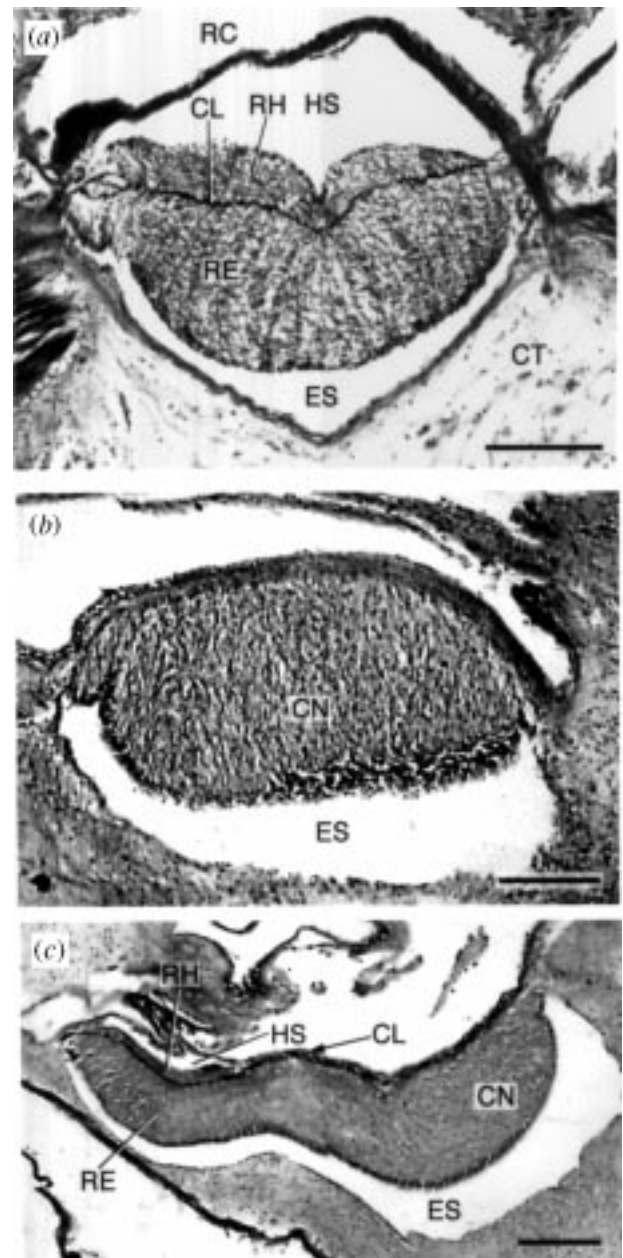
the coelomic epithelium covering the circular muscles. In cross-sections of the circular muscles the labelled fibres were observed at intervals (see figure 3*j*).

(ii) *Tube feet*

NGIWYa-LI was detected in the podial nerve where it emerged from the radial nerve cord (see figure 3*k*) and throughout its length (see figure 3*l*). NGIWYa-LI was also observed in nerve plexi lining the tube feet (see figure 3*l*), however, cell bodies could not be detected.
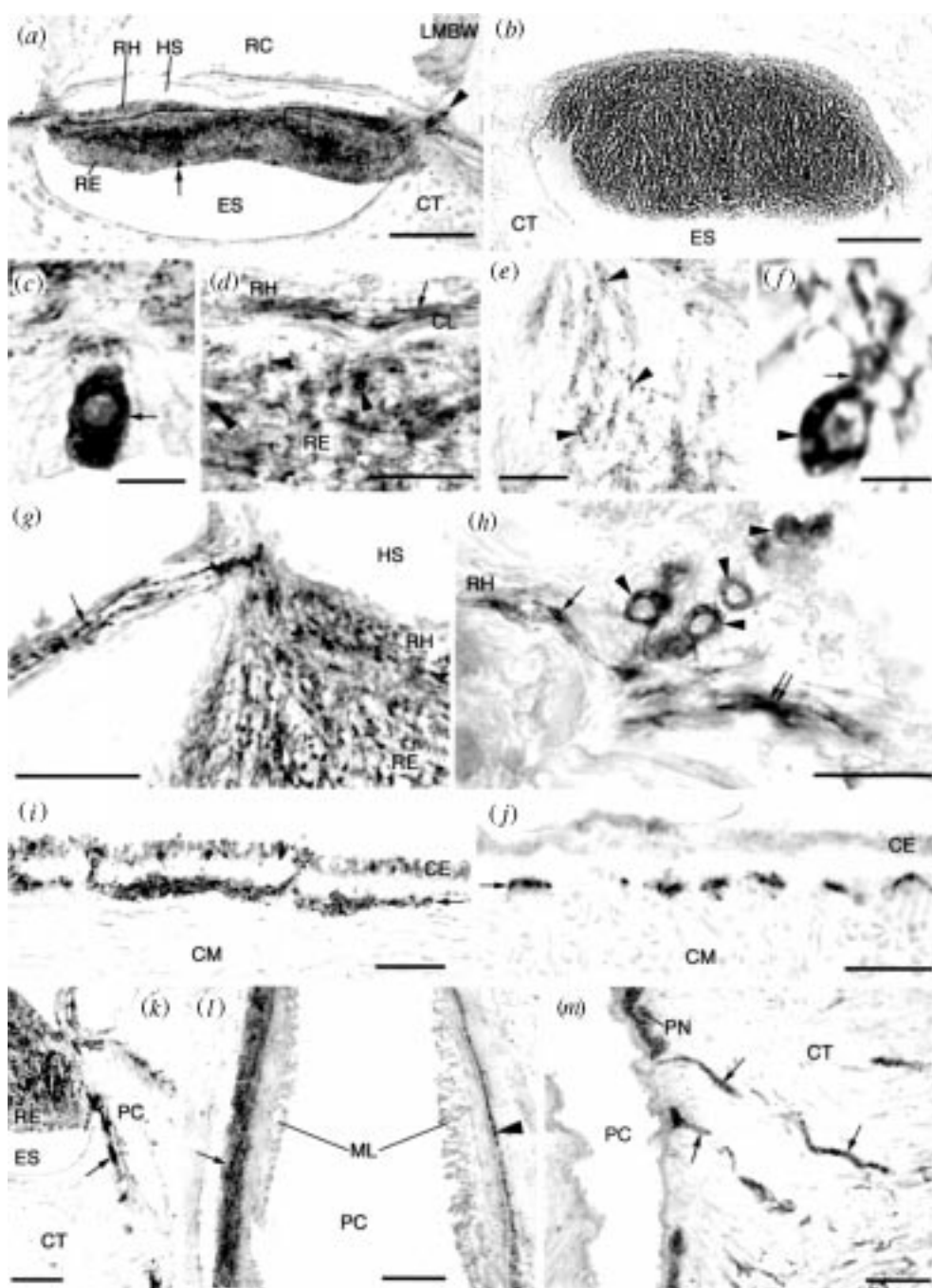
Figure 3. NGIWYamide immunoreactivities in the radial nerve cord, circumoral nerve ring and tube foot. (*a*) NGIWYa-LI in the radial nerve cord. Details of the ectoneural cell body (arrow), connective tissue portion (square) and cell bodies in a branching region (arrowhead) are shown in (*c*), (*d*) and (*h*), respectively; scale bar, 100 μm. (*b*) NGIWYa-LI in the circumoral nerve ring; scale bar, 100 μm. (*c*) A cell body expressing NGIWYa-LI (arrow) located in the periphery of the ectoneural nerve of the radial nerve; scale bar, 5 μm. (*d*) Cell bodies expressing NGIWYa-LI (arrowheads) in the ectoneural portion near the connective tissue layer separating the hyponeural and ectoneural parts. Fibres of the hyponeural nerve have NGIWYa-LI (arrow); scale bar, 20 μm. (*e*) Fibres and varicosities with NGIWYa-LI (arrowheads) in the ectoneural part of the radial nerve; scale bar, 30 μm. (*f*) A cell body expressing NGIWYa-LI (arrowhead) in the mid portion of the circumoral nerve ring. An axon-like process (arrow) can be seen; scale bar, 5 μm. (*g*) Varicose nerve fibres expressing NGIWYa-LI (arrow) in the branch extending from the hyponeural nerve towards the circular muscle of the body wall (to the left); scale bar, 50 μm. (*h*) Cell bodies expressing NGIWYa-LI (arrowheads) located at the coelomic epithelium where a branch emerges from the hyponeural nerve towards the circular muscle. Axon-like processes from these cell bodies can be seen. Some of them seem to run towards the hyponeural nerve (arrow) and some towards the circular muscle (double arrow); scale bar, 20 μm. (*i*) Length section of fibres expressing NGIWYa-LI (arrow) located in the coelomic epithelium (CE) lining the circular muscle; scale bar, 50 μm. (*j*) Cross-section of fibres expressing NGIWYa-LI (arrow) located in the coelomic epithelium lining the circular muscle. These fibres run parallel at certain intervals; scale bar, 50 μm. (*k*) NGIWYa-LI (arrow) in the podial nerve extending from the ectoneural part of the radial nerve; scale bar, 50 μm. (*l*) Fibres expressing NGIWYa-LI (arrow) in the podial nerve and NGIWYamide-positive nerve plexus (arrowhead) lining the tube foot; scale bar, 100 μm. ML, muscle layer. (*m*) Fibres expressing NGIWYa-LI (arrows) emerge from the podial nerve and extend into the connective tissue of the body wall dermis; scale bar, 50 μm. Additional abbreviations as in figure 1.
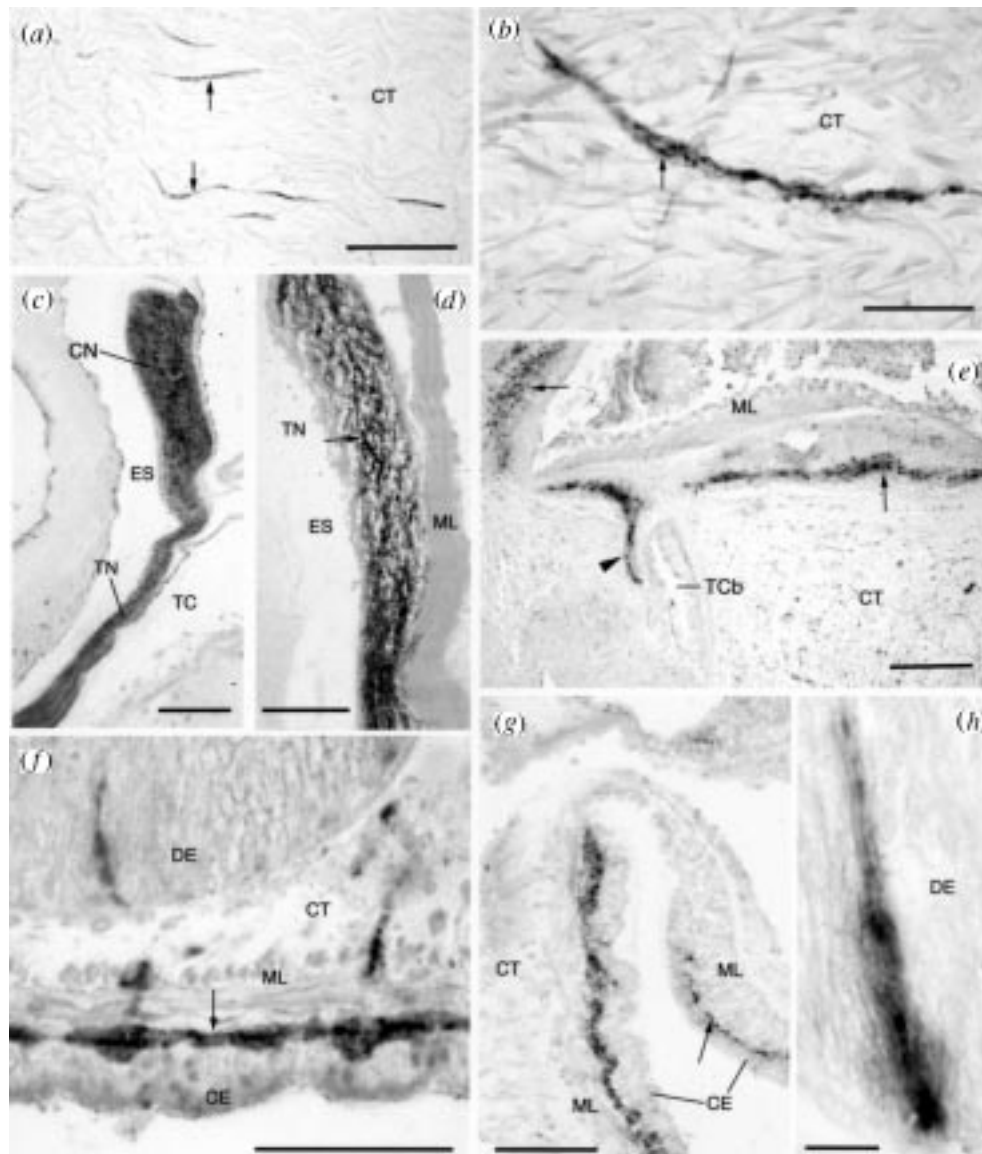
Figure 4. NGIWYamide immunoreactivities in the body wall dermis, tentacles and intestine. (*a*) Cellular processes expressing NGIWYa-LI (arrows) in the connective tissue of the body wall dermis; scale bar, 100 μm. (*b*) Detail of (*a*). Notice the varicosities of the cellular process with NGIWYa-LI (arrow); scale bar, 50 μm. (*c*) NGIWYa-LI in the circumoral nerve ring and in the tentacular nerve branching off from the nerve ring; scale bar, 50 μm. (*d*) Fibres expressing NGIWYa-LI (arrow) in the tentacular nerve; scale bar, 50 μm. (*e*) Nerve fibres with NGIWYa-LI (arrowhead) branching from the main nerve trunk of a tentacle (arrows) run along the water canal of the terminal bud of the tentacle (TCb). The arrowhead indicates a branching point. The direction of the tentacular tip is towards the bottom; scale bar, 5 μm. (*f*) Basiepithelial nerve plexus expressing NGIWYa-LI (arrow) in the intestine; scale bar, 200 μm. DE, digestive epithelium. (*g*) Basiepithelial nerve plexus expressing NGIWYa-LI (arrow) in the mesentery connecting the intestine and body wall; scale bar, 100 μm. (*h*) NGIWYa-LI in the digestive epithelium; scale bar, 50 μm. Additional abbreviations as in figure 1.

(iii) *Dermis*

Cellular processes with NGIWYa-LI were abundant in the body wall dermis (see figure 4*a*). They carried numerous varicosities (see figure 4*b*). NGIWYamide-positive fibres branched off from the podial nerve and penetrated into the connective tissue of the body wall dermis (see figure 3*m*), but not into the tube feet dermis.

(iv) *Tentacles*

Each tentacle branches into numerous terminal buds; the water canal and the accompanying tentacular nerve ramify correspondingly. NGIWYa-LI was detected in the tentacular nerve where it emerged from the circumoral nerve ring (see figure 4*c*) and throughout its length running along the water canal of the tentacle stalk (see

figure 4*d,e*). Nerve fibres with NGIWYa-LI branching from the tentacular nerve ran along the water canal branches of terminal buds (see figure 4*e*). Immunoreactive cell bodies were not observed in tentacular nerves.

(v) *Intestine*

From the lumen towards the coelom, the wall of the digestive tract consists of a single digestive epithelium, a connective tissue layer and a muscular mesothelium (Feral & Massin 1982). NGIWYa-LI was localized predominantly in the basiepithelial nerve plexus of the coelomic epithelium (see figure 4*f*) which makes up the mesothelium of Feral & Massin (1982). The mesentery connecting the digestive tract to the body wall also contained a NGIWYamide-positive nerve plexus (see
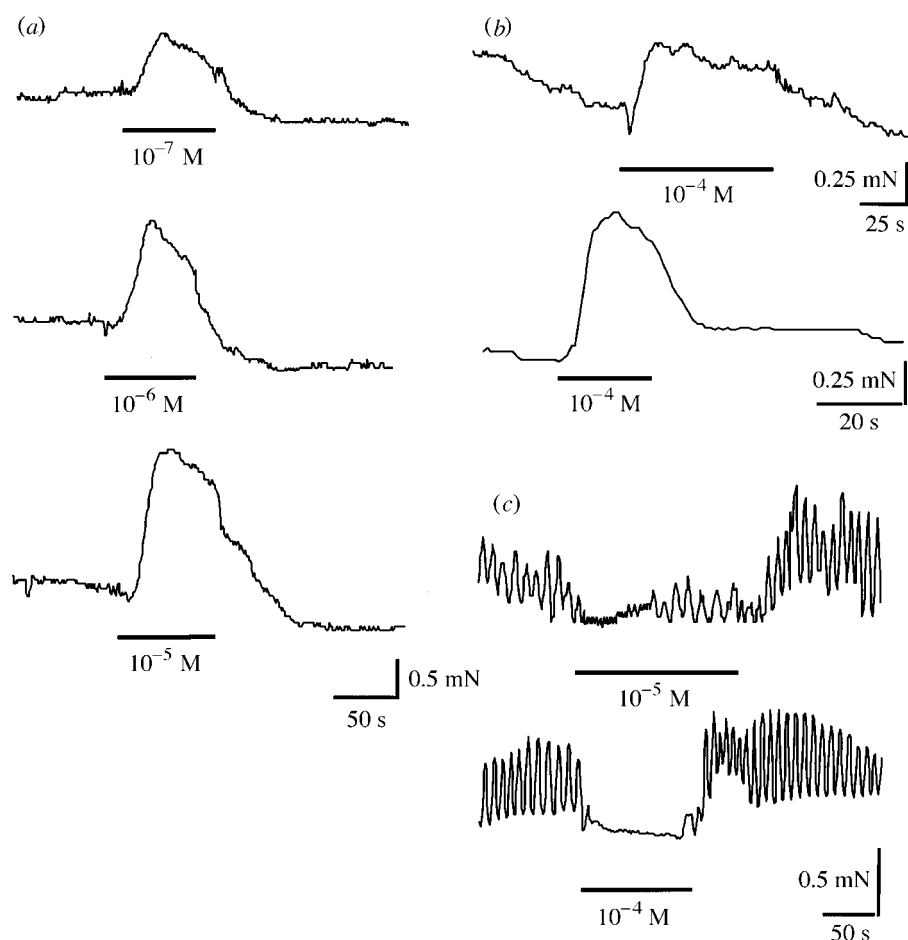
Figure 5. Effects of NGIWYamide on musculature. (*a*) NGIWYamide-induced contraction of the longitudinal body wall muscle. (*b*) NGIWYamide-induced contraction of a tentacle. (*c*) NGIWYamide inhibition of spontaneous contraction of the intestine. The horizontal bar indicates application of the drug.

figure 4*g*). NGIWYa-LI was observed in some cells of the digestive epithelium (see figure 4*h*).

(vi) *Pre-absorption test*

In control experiments, pre-absorption of NGIWYamide antisera with 10^{-3} M NGIWYamide abolished staining completely and with 10^{-6} M NGIWYamide immunoreactivity was much weaker with only a few single spots of immunoreactivity.

(c) *Pharmacology*

(i) *Longitudinal muscles of the body wall*

NGIWYamide induced contraction of the longitudinal muscles of the body wall at concentrations of 10^{-7} M or higher (see figure 5*a*). Tension developed within seconds after application of the peptide, reached a maximum in *ca.* 50 s and then decreased gradually. The muscle relaxed when the peptide was washed out. Higher doses evoked larger contractions indicating a dose-dependent response.

(ii) *Tentacles*

The effect of 10^{-6} M to 10^{-4} M NGIWYamide on tentacles was studied. Contraction was observed only at 10^{-4} M (see figure 5*b*). Tension developed within seconds and reached a maximum *ca.* 20 s after application.

(iii) *Intestine*

The intestine showed spontaneous contraction. Concentrations of 10^{-7} M and 10^{-6} M NGIWYamide did not have any effect on this spontaneous rhythm, whereas 10^{-5} M and 10^{-4} M concentrations inhibited the contractions. The inhibitory effect was weak at 10^{-5} M, but complete inhibition was observed at 10^{-4} M NGIWYamide (see figure 5*c*). The inhibition was observed within seconds and continued during application of the peptide. After washing the peptide out, spontaneous contraction recovered within seconds.

In some cases during application or washing out of NGIWYamide the force dropped to some degree due to the effects of the water flow regime.

4. DISCUSSION

The present study revealed the widespread distribution of NGIWYamide throughout the holothurian nervous system. This is good evidence that NGIWYamide is a neuropeptide in sea cucumbers. Because of its wide distribution in nerves, NGIWYamide immunostaining could be used as a method for specifically staining even small nerves which are notoriously difficult to identify with common methods. We could follow the innervation route to muscles and to the dermis with this protocol.

NGIWYamide is one of the few neuropeptides known in echinoderms. In previous studies, GFNSALMFamide (S1) and SGPYSFNSGLTFamide (S2) have been isolated from the radial nerve cord of the sea star *Asterias rubens* (Elphick *et al.* 1991) and SGYSVLYFamide and GFSKLYFamide have been isolated from the digestive tract of the sea cucumber *H. glaberrima* (Diaz-Miranda *et al.* 1992). They are members of a SALMFamide family of neuropeptides showing a

common periodic sequence including serine, leucine and phenylalanine. NGIWYamide does not fit into this pattern and, thus, was classified as a novel neuropeptide which is different from currently known echinoderm neuropeptides (Birenheide *et al.* 1998). Here, we will discuss our findings in comparison with neuropeptides of the SALMFamide family and we will interpret the innervation of holothurian tissues by hyponeural and ectoneural nerves as revealed through NGIWYamide immunostaining.

(a) *Radial nerve and circumoral nerve ring*

The circumoral nerve ring has been reported to consist of the ectoneural nervous system only (Hyman 1955; Cobb 1987), a feature confirmed by this study. The circumoral nerve ring contains fibres and cells with NGIWYa-LI. S1-like immunoreactivities were found in the circumoral nerve ring of the sea star *A. rubens* (Newman *et al.* 1995*a*), the brittle star *Ophiura ophiura* (Ghyoot *et al.* 1994) and in the swellings of the circumoral nerve ring of the brittle star *Amphipholis squamata* (Bremaeker *et al.* 1997). The present study is the first to show the presence of neuropeptides in fibres and cell bodies of the circumoral nerve ring in holothurians.

In this study, NGIWYa-LI was abundant in the nerve fibres and cell bodies in the radial nerves. Positive labelling in the radial nerve was also found with antibodies against GFSKLYFamide in the sea cucumber *H. glaberrima* (Diaz-Miranda *et al.* 1995), against S1 and S2 in the sea star *A. rubens* (Newman *et al.* 1995*a*) and in the brittle star *A. squamata* (Bremaeker *et al.* 1997) and against S1 in the brittle star *O. ophiura* (Ghyoot *et al.* 1994). This association of SALMFamides as well as NGIWYamide with the echinoderm nervous system points to an important role for these neuropeptides. In the light of our physiological data it can be speculated that NGIWYamide and possibly other peptides serve as neurotransmitters in the echinoderm nervous system. This sheds new light on the still unknown mode of transmission in echinoderm nerves which are lacking synapses (Cobb 1987).

(b) *Circular muscle and the longitudinal muscles of the body wall*

Hyman (1955) suggested that the hyponeural system should be regarded mainly or exclusively as a motor system supplying the muscle fibres of the body wall. This speculation is supported by our finding that nerve fibres with NGIWYa-LI extend from the hyponeural portion of the radial nerve cord towards the body wall and appear to innervate the circular muscle. That NGIWYamide induced contraction of the longitudinal muscles of the body wall suggests peptidergic control of the longitudinal muscles of the body wall, although no NGIWYa-LI was found inside or on the muscle bundles of the longitudinal muscles of the body wall. The longitudinal muscles of the body wall may be controlled by fibres containing NGIWYamide, either directly or indirectly. In the case of direct control, the apparent absence of NGIWYa-LI in the longitudinal muscles of the body wall might be caused by the following reasons: NGIWYamide-containing fibres may be too small to be detected, the innervation may be confined to a very small region or there may be a novel mechanism of neuromuscular transmission such as non-synaptic, wide-range or long-distance

release of NGIWYamide. In spite of this, our results suggest that NGIWYamide certainly plays some role in muscle control. In another sea cucumber species GFSKLYFamide-like immunoreactive fibres seem to innervate body wall muscles because they extended from the radial nerve cord towards the muscles and immunoreactivity was observed inside the muscle bundles (Diaz-Miranda *et al.* 1995). Contrary to NGIWYamide, however, GFSKLYFamide induced relaxation of the longitudinal muscles of the body wall (Diaz-Miranda & Garcia-Arraras 1995). From these results, we conclude that holothurians possess several neuropeptides for neuromuscular transmission and modulation, some of which may work antagonistically. This forms the basis for sophisticated control of effector organs.

(c) *Tube feet and tentacles*

The distribution of NGIWYa-LI in the podial and tentacular nerves, together with the contraction of tentacles induced by this peptide, suggests that NGIWYamide has controlling functions in feeding and locomotion. Labelling with antibodies against SALMFamide neuropeptides was seen in the tentacular and podial nerves of the sea cucumber *H. glaberrima* (Diaz-Miranda *et al.* 1995), in the podial nerves ('longitudinal nerves') and basal nerve ring of the tube feet of the sea star *A. rubens* (Newman *et al.* 1995*a*) and in the podial ganglion, podial nerves ('longitudinal nerves') and basal nerve ring of the tube foot of the brittle star *A. squamata* (Bremaeker *et al.* 1997). However, physiological data are scarce. Only Elphick *et al.* (1995) reported that neither S1 or S2 have any effect on asteroid tube feet. Our finding that NGIWYamide affects tentacles is therefore the first account of an effect of neuropeptides on the water vascular system.

(d) *Intestine*

NGIWYa-LI in the basiepithelial nerve plexus of the intestinal coelomic epithelium and our physiological data indicate that NGIWYamide has some role in modulating intestinal muscular activities. As shown by the positive labelling of the mesentery nerve plexus, innervation is probably from the radial nerve via the mesentery. NGIWYamide-positive cells in the digestive epithelium layer suggest that NGIWYamide might also be involved in the control of digestive secretion or uptake.

Neuropeptide immunoreactivities have been reported before from intestinal tissues of other echinoderms. For example, GFSKLYFamide-positive cells were found in the basiepithelial nerve plexus, muscle layer, connective tissue layer and digestive epithelium of the intestine of the sea cucumber *H. glaberrima* in physiological tests, while GFSKLYFamide relaxed the intestine *in vitro* (Diaz-Miranda *et al.* 1995). In the digestive tract of the sea star *A. rubens*, S2-like immunoreactivity was localized in the basiepithelial nerve plexus and in the digestive epithelium (Newman *et al.* 1995*b*). S2 caused relaxation of contraction induced by ACh in the cardiac stomach (Elphick *et al.* 1995). From these data we conclude that the basiepithelial nerve plexus of the coelomic epithelium and its neuropeptides play an important role in controlling the muscular activities of the intestine, similar to their role in controlling the muscular activities of the body wall. The body wall muscles and the intestinal muscles probably derive from

the same coelomic epithelium, i.e. the epithelium of the main body cavity. Thus, it is not surprising that the same neuropeptide is found and is functional in these two muscular tissues. Besides muscular control, neuropeptides might also influence the secretion and uptake via the cells of the digestive epithelium, but physiological data of such activities are still lacking.

(e) *Connective tissue of the dermis*

Birenheide *et al.* (1998) reported that NGIWYamide stiffened the connective tissue of the dermis in the sea cucumbers *Holothuria leucospilota* and *A. japonicus*. It was suggested that NGIWYamide had its effect through the nervous system, because the effect was suppressed by anaesthetics. This suggestion is supported by our present observations: fibres with NGIWYa-LI extended from the ectoneural podial nerve into the body wall dermis and NGIWYa-LI was found in numerous cellular processes scattered in the dermis. Some of these cellular processes may connect with the fibres extending from the podial nerve although direct connection was not observed in the present study. These cellular processes seem to secrete neurotransmitters or modulators for controlling catch connective tissue directly or indirectly, because they have numerous varicosities. These results suggest that catch connective tissue in the dermis is directly controlled by the ectoneural nervous system. Motokawa (1982) reported that granule-filled cells with long slender processes are frequently found in the dermis. The size and shape of these cells resemble the cells stained in our present study which raises the possibility that they may be identical. Diaz-Miranda *et al.* (1995) reported that some cell bodies and nerve fibres labelled with GFSKLYFa-IL were found within the connective tissue matrix of the body wall of the sea cucumber *H. glaberrima*. However, they did not report whether these labelled cells are connected to hyponeural or ectoneural systems. Our data give the first evidence that the connective tissue of sea cucumbers is innervated by the ectoneural nervous system.

In summary, the present investigation has shown that NGIWYamide isolated from sea cucumbers is a neuropeptide located in the nervous system and is involved in the control of musculature and the catch connective tissue of sea cucumbers. Our data, together with those of former authors, clearly suggest that peptidergic neurons are significant components of the echinoderm nervous system and play important roles in the motor control of echinoderms. The possible roles of neuropeptides include neurotransmission and modulation. Further investigation of neuropeptides will be an important clue in understanding the enigmatic nervous system of echinoderms.

REFERENCES

Birenheide, R., Tamori, M., Motokawa, T., Ohtani, M., Iwakoshi, E., Muneoka, Y., Fujita, T., Minakata, H. & Nomoto, K. 1998 Peptides controlling stiffness of connective tissue in sea cucumbers. *Biol. Bull.* **194**, 253–259.

Bremaeker, N. D., Deheyn, D., Thorndyke, M. C., Baguet, F. & Mallefet, J. 1997 Localization of S1- and S2-like immuno-reactivity in the nervous system of the brittle star *Amphipholis squamata* (Delle Chiaje 1828). *Proc. R. Soc. Lond.* B **264**, 667–674.

Cobb, J. L. S. 1987 Neurobiology of the Echinodermata. In *Invertebrate nervous systems* (ed. M. A. Ali), pp. 483–526. New York: Plenum Press.

Diaz-Miranda, L. & Garcia-Arraras, J. E. 1995 Pharmacological action of the heptapeptide GFSKLYFamide in the muscle of the sea cucumber *Holothuria glaberrima* (Echinodermata). *Comp. Biochem. Physiol.* **110C**, 171–176.

Diaz-Miranda, L., Price, D. A., Greenberg, M. J., Lee, T. D., Doble, K. E. & Garcia-Arraras, J. E. 1992 Characterization of two neuropeptides from the sea cucumber *Holothuria glaberrima. Biol. Bull.* **182**, 241–247.

Diaz-Miranda, L., Blanco, R. E. & Garcia-Arraras, J. E. 1995 Localization of heptapeptide GFSKLYFamide in the sea cucumber *Holothuria glaberrima* (Echinodermata): a light and electron microscopic study. *J. Comp. Neurol.* **352**, 626–640.

Elphick, M. R., Price, D. A., Lee, T. D. & Thorndyke, M. C. 1991 The SALMFamides: a new family of neuropeptides isolated from an echinoderm. *Proc. R. Soc. Lond.* B **243**, 121–127.

Elphick, M. R., Newman, S. J. & Thorndyke, M. C. 1995 Distribution and action of SALMFamide neuropeptides in the starfish *Asterias rubens. J. Exp. Biol.* **198**, 2519–2525.

Feral, J. & Massin, C. 1982 Digestive system: Holothuroidea. In *Echinoderm nutrition* (ed. M. Jangoux & J. Lawrence), pp. 191–213. Rotterdam: A. A. Balkema.

Ghyoot, M., Cobb, J. L. S. & Thorndyke, M. C. 1994 Localization of neuropeptides in the nervous system of the brittle star *Ophiura ophiura. Phil. Trans. R. Soc. Lond.* B **346**, 433–444.

Humason, G. L. 1979 *Animal tissue technique.* San Francisco: W. H. Freeman & Co.

Hyman, L. H. 1955 *The invertebrates: Echinodermata*, vol. 4. New York: McGraw-Hill.

Iwakoshi, E., Ohtani, M., Takahashi, T., Muneoka, Y., Ikeda, T., Fujita, T., Minakata, H. & Nomoto, K. 1995 Comparative aspects of structure and action of bioactive peptides isolated from the sea cucumber *Stichopus japonicus*. In *Peptide chemistry 1994* (ed. M. Ohno), pp. 261–264. Osaka: Protein Research Foundation.

Motokawa, T. 1981 The stiffness change of the holothurian dermis caused by chemical and electrical stimulation. *Comp. Biochem. Physiol.* **70C**, 41–48.

Motokawa, T. 1982 Fine structure of the dermis of the body wall of the sea cucumber, *Stichopus chloronotus*, a connective tissue which changes its mechanical properties. *Galaxea* **1**, 55–64.

Motokawa, T. 1984 The viscosity change of the body-wall dermis of the sea cucumber *Stichopus japonicus* caused by mechanical and chemical stimulation. *Comp. Biochem. Physiol.* **77A**, 419–423.

Newman, S. J., Elphick, M. R. & Thorndyke, M. C. 1995*a* Tissue distribution of SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. 1. Nervous and locomotory systems. *Proc. R. Soc. Lond.* B **261**, 139–145.

Newman, S. J., Elphick, M. R. & Thorndyke, M. C. 1995*b* Tissue distribution of SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. 2. Digestive system. *Proc. R. Soc. Lond.* B **261**, 187–192.

Peerters, J. M., Hazendonk, T. G., Beuvery, E. C. & Tesser, G. I. 1989 Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J. Immunol. Meth.* **120**, 133–143.

Ruppert, E. D. & Barnes, R. D. (eds) 1994 Mutable connective tissue. In *Invertebrate zoology*, pp. 921–922. Fort Worth: Saunders College Publishing.

Schmidt-Nielsen, K. (ed.) 1997 Catch connective tissue. In *Animal physiology*, p. 430. Cambridge University Press.